Assessment of the role of early life exposure to endocrine disrupting compounds in programming of obesity in a mouse model

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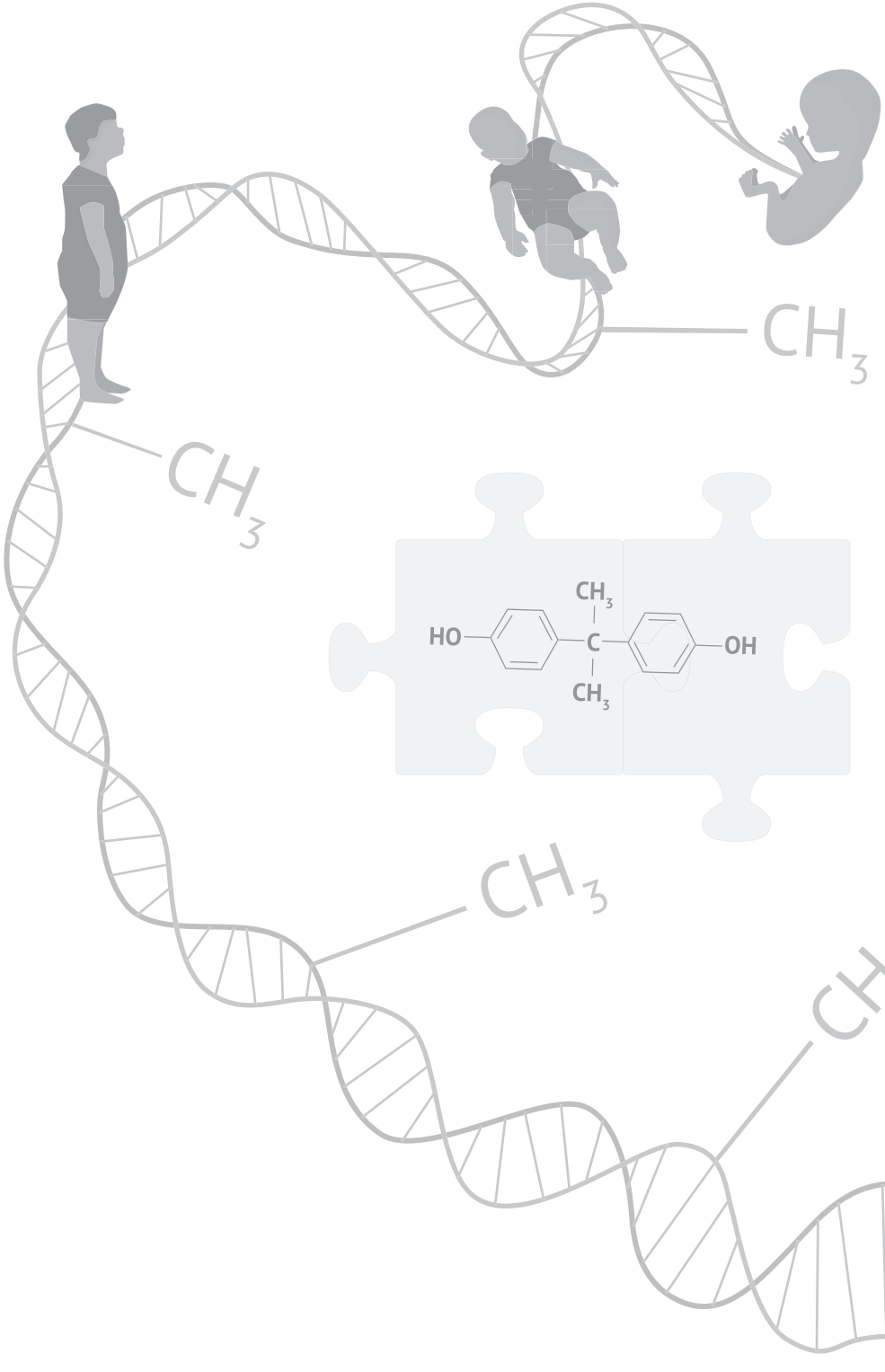
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Chapter 3

Liver DNA methylation analysis in adult female C57BL/6JxFVB mice following perinatal exposure to bisphenol A

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Abstract

Bisphenol A (BPA) is a compound released from plastics and other consumer products used in everyday life. BPA exposure early in fetal development is proposed to contribute to programming of chronic diseases like obesity and diabetes, by affecting DNA methylation levels. Previously, we showed that *in utero* and lactational exposure of C57BL/6JxFVB hybrid mice *via* maternal feed using a dose range of 0–3000 μg/kg body weight/day (μg/kg bw/d) resulted in a sex-dependent altered metabolic phenotype in offspring at 23 weeks of age. The most univocal effects were observed in females, with reduced body weights and related metabolic effects associated with perinatal BPA exposure. To identify whether the effects of BPA in females are associated with changes in DNA methylation, this was analyzed in liver, which is important in energy homeostasis. Measurement of global DNA methylation did not show any changes. Genome-wide DNA methylation analysis at specific CpG sites in control and 3000 μg/kg bw/d females with the digital restriction enzyme analysis of methylation (DREAM) assay revealed potential differences, that could, however, not be confirmed by bisulfite pyrosequencing. Overall, we demonstrated that the observed altered metabolic phenotype in female offspring after maternal exposure to BPA was not detectably associated with liver DNA methylation changes. Still, other tissues may be more informative.

Introduction

Bisphenol A (BPA) is used as a monomer in the production of polycarbonate plastics and epoxy resins. It is present in many consumer products from which it can leach, such as plastic water bottles, food containers, can linings, and thermal paper (Brotons et al., 1995). Since BPA is ubiquitously present in the environment, humans are continuously exposed to low levels of BPA, mainly via the oral route (Casals-Casas and Desvergne, 2011).

The safe level for humans, the tolerable daily intake, is based on liver effects in rodent multigenerational toxicity studies using a wide dose range. However, more subtle effects may be caused by the endocrine activity of BPA, particularly its estrogenic activity, which may occur through high affinity binding to the estrogen related receptor-gamma (Babu et al., 2012) rather than through its low potency effect via estrogen receptor activation (Ben-Jonathan and Steinmetz, 1998). In animal and epidemiological studies, low dose levels of BPA have been linked to health effects such as cancer (reviewed by Soto and Sonnenschein, 2010), cardiovascular disease (Yan et al., 2013), metabolic syndrome (Teppala et al., 2012), diabetes (Alonso-Magdalena et al., 2010), and asthma (Vaidya and Kulkarni, 2012). Variable and inconsistent results between studies, however, nourish the controversy over the link of low dose BPA to disease (Bolt and Stewart, 2011; Casey and Neidell, 2013; Rochester, 2013; Vandenberg et al., 2009).

In humans, environmental exposure to low doses of BPA already starts *in utero* as BPA can cross the placenta (Schonfelder et al., 2002) and continues postnatally as it is present in breast milk (Otaka et al., 2003) and plastic baby bottles (Vandenberg et al., 2007). During this highly sensitive early life period, following the developmental origins of health and disease paradigm (Gluckman and Hanson, 2004), exposure to external factors, including environmental contaminants, can result in disrupted programming of the organism (Gluckman et al., 2005; Vickers, 2014). This disrupted programming can ultimately predispose an individual to chronic diseases later in life, including metabolic disorders (Oken and Gillman, 2003).

A growing number of studies indicate epigenetic modifications through DNA methylation as a mechanism for disrupted fetal metabolic programming (Barres and Zierath, 2011; Ruchat et al., 2013). BPA exposure has been shown to affect the level

of DNA methylation in rodent studies and the nature of these methylation changes depends on species, strain, sex, dose, loci, and tissue studied (Table 1). In prenatally treated mice, methylation differences at the estrogen receptor α (ERα), activated by BPA, were dependent on sex and brain region (Kundakovic et al., 2013). These authors observed an increase in methylation of male prefrontal cortex tissue and a decrease in methylation of female hypothalamic tissue. Similarly, sex-dependent effects were observed in mice treated prenatally through adulthood with BPA (Patel et al., 2013). An increase in global DNA methylation was found in ventricle heart in males, whereas females showed a decrease. Gestational BPA exposure resulted in age-dependent DNA methylation differences in mammary glands of rat pups (Dhimolea et al., 2014). In perinatal BPA exposure studies with Agouti mice, global DNA methylation of DNA isolated from the tail increased (Anderson et al., 2012), while global methylation of bone marrow-derived mast cells decreased (O'Brien et al., 2014). In addition, methylation was decreased at the Agouti promoter (Dolinoy et al., 2007) and liver showed altered regions of methylation (Kim et al., 2014). Furthermore, perinatal exposure to BPA decreased global DNA methylation both in mice placenta (Susiarjo et al., 2013) and in liver of rat offspring (Ma et al., 2013). In liver of rat offspring, the promoter of glucokinase, a gene important for insulin resistance and glucose intolerance, showed hypermethylation (Ma et al., 2013) which persisted into the F2 generation (Li et al., 2014). However, *Hoxa10*, a gene expressed in the uterus and critical for its development (Bromer et al., 2010) and *Igf2-H19*, an imprinted control region tested in sperm (Doshi et al., 2013), were hypomethylated.

In a previous study, we investigated whether early life exposure to BPA programs for obesity and metabolic impairment (van Esterik et al., 2014). C57BL/6JxFVB hybrid mice were exposed to BPA during gestation and lactation via maternal feed using a dose range of 0–3000 μg/kg body weight/day (μg/kg bw/d). Perinatal BPA exposure resulted in an altered metabolic phenotype of offspring at 23 weeks of age. The phenotype was sex-dependent, and most reliably detected in females, because the effects in males were possibly confounded by litter size. Female progeny showed a decrease of body weight, and a decrease in weight of fat pads and adipocyte cell size potentially explaining the decrease in body weight. In addition, free fatty acid, triglyceride, adiponectin and leptin levels were changed. As effects were observed in female adults while BPA exposure was terminated at an early age (3 weeks), the effects were programmed during early developmental stages, compatible with the developmental origins of health and disease paradigm. To identify whether the metabolic changes observed

from perinatal exposure to BPA were programmed through epigenetic modifications, liver DNA methylation in adult female offspring was measured. Liver was selected as the target organ since it is a key player in energy and xenobiotic metabolism, can be easily accessed, and is a relatively homogenous tissue. As a first screen, global DNA methylation was measured with an HPLC method. Furthermore, to identify specific targets, DNA methylation at CpG sites recognized by the restriction enzyme SmaI was measured on a genome-wide scale using the DREAM assay (digital restriction enzyme analysis of methylation) (Jelinek et al., 2012). Sites found differentially methylated in the DREAM assay were validated with bisulfite pyrosequencing.

Methods

Animals and perinatal exposure

Animal study design is described in detail in van Esterik et al. (2014). Briefly, nulliparous female C57BL/6J mice (Charles River, Sulzfeld, Germany) were fed a NIH-07 diet containing BPA starting 2 weeks before mating through to weaning age of their offspring. For every dose group, four females were mated with two previously non-exposed male FVB mice (GPL, Bilthoven, The Netherlands) for one week during which time the males received the same diet as the females they were mated with. BPA concentrations in feed were 0, 0.017, 0.056, 0.17, 0.56, 1.7, 5.6, and 16.7 mg/kg, which corresponded to 0, 3, 10, 30, 100, 300, 1000, and 3000 μg/kg bw/d. Offspring were weaned at 3 weeks of age and on average 8 mice per sex (range 4–10, evenly recruited from available litters) were included for follow-up. All weaned offspring were fed the control NIH-07 diet. From 17 weeks until the end of the study, females were challenged for an obesogenic response with a high fat diet. Both feed and water were supplied *ad libitum* throughout the study. At the age of 23 weeks, after being fasted for 16 h, mice were sacrificed. Livers were snapfrozen in liquid nitrogen and stored at –80 °C. This study was approved by the Animal Experimentation Ethical Committee of our institute under permit number 200900208, and carried out in accordance with prevailing legislation.

Table 1. DNA methylation effects from BPA exposure

Strain species	Dose µg/kg bw/d	Exposure route	Exposure window	Age tested	Tissue	Assay	Methylation result [2]	Reference
Agouti mouse	10,000 [1]	Diet	2w pre-mating–PND22	PND22	Tail	Bisulfite sequencing	↓ At *Agouti* promoter	Dolinoy et al. (2007) [3]
Agouti mouse	0.01–10–10,000 [1]	Diet	2w pre-mating–PND22	PND22	Tail	LUMA; bisulfite sequencing	↑ Global with all doses; ↓ at *Agouti* promoter with 10,000	Anderson et al. (2012) [3]
Agouti mouse	10–10,000	Diet	2w pre-mating–PND22	PND22	Liver	M-NGS, Sequenom EpiTYPER MassARRAY	Enrichment of RAMs, ↑ at *Myh7b* promoter with both doses; ↓ at *Slc22a12* promoter with 10	Kim et al. (2014) [3]
Agouti mouse	0.01–10–10,000 [1]	Diet	2w pre-mating–PND21	6m	Bone marrow-derived mast cells	Enzyme immunoassay kit	↓ Global [5]	O'Brien et al. (2014) [3]
CD-1 mouse	5000	i.p.	GD9–16	2w	Uterus	Bisulfite sequencing	F: ↓ at *Hoxa10* promoter + intron	Bromer et al. (2010)
BALB/c mouse	2–20–200	Oral gavage	GD0–19	PND28	Brain	Bisulfite sequencing	M: ↑ at ERα in prefrontal cortex with 20; F: ↓ at ERα in hypothalamus with 20	Kundakovic et al. (2013)
Wistar rat	50	Oral gavage	GD0–PND21	3w; 21w	Liver	HPLC; bisulfite sequencing	M: ↓ global; ↑ at *Gck* promoter	Ma et al. (2013) [4]
SD rat	40	Oral gavage	GD0–PND21	16w (F1); 22w (F2)	Sperm (F1); liver (F2)	Methylamp TM global Quantification Kit; bisulfite sequencing	M: ↓ global, ↑ at *Gck* promoter (sperm + liver)	Li et al. (2014) [4]

Table 1. Continued

Strain species	Dose µg/kg bw/d	Exposure route	Exposure window	Age tested	Tissue	Assay	Methylation result [2]	Reference
Holtzman rat	400	s.c.	PND1–5 (F1)	PND75 (F1); GD20 (F2)	Sperm (F1); resorbed embryo (F2)	BSP, cloning and sequencing	↓ At *Igf2–H19* (sperm); altered methylation pattern at *Igf2–H19* (resorbed embryo)	Doshi et al. (2013)
C57BL/6n mouse	0.5–5–200	Drinking water	GD11–4m (0.5-5) GD11–PND21 (200)	4m	Ventricle heart	MethylFlash ELISA kit; bisulfite sequencing	M: ↑ global with 0.5; F: ↓ global with 0.5; site, sex, and dose-dependent effects at CASQ2	Patel et al. (2013)
C57BL/6xC7 mouse	10–10,000	Diet	2w pre-mating-E9.5/ E12.5	GD9.5; GD12.5	Placenta	LUMA	↓ Global with 10,000	Susiarjo et al. (2013)
Wistar-Furth rat	250	s.c. osmotic pumps	GD9–PND1	PND4; PND21; PND50	Mammary gland	Nimblegen ChIP array	F: site and age-dependent effects throughout genome	Dhimolea et al. (2014)
C57BL/6JxFVB mouse	3–10–30–100–300–1000–3000 3000	Diet	2w pre-mating–PND21	23w	Liver	HPLC DREAM; pyrosequencing	F: no change	This study

GD/PND, gestation/postnatal day; global, global DNA methylation; i.p., intraperitoneal; s.c., subcutaneously; M/F, male/female; months; w, weeks; BSP, bisulfite genomic sequencing; DREAM, Digital Restriction Enzyme Analysis of Methylation; LUMA, Luminometric Methylation Assay; M-NGS, MethylPlex-Next Generation Sequencing; RAM, region of altered methylation.

[1] Conversion of dose in mg/kg diet to µg/kg bw/d based on Susiarjo et al. (2013).

[2] When sex is not indicated, males and females are mixed.

[3,4] Same letter indiciates studies have been performed by the same research group.

[5] Significant effect for dose 0.01; dose 10,000 was nearly significant ($p = 0.057$) and a similar trend was observed for dose 10.

DNA extraction

Livers were selected representing equal numbers of individuals from as many different litters as available per dose group (range 2–4 litters/dose group). Livers were finely ground using a mortar and pestle in liquid nitrogen. Then, for analysis of global methylation and pyrosequencing, DNA was extracted with the DNeasy Blood and Tissue kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions and stored at –20 °C until further analysis. An additional RNA cleanup step was added prior to DNA extraction by treatment of lysates with 1 mg/mL RNAse A (Roche, Basel, Switzerland) for 5 min at room temperature. For the DREAM assay, DNA was extracted from homogenized livers with the ZR-Duet DNA/RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions and stored at –20 °C. DNA was eluted with nuclease-free water and DNA concentration was determined using a NanoDrop spectrophotometer (Isogen Life Science, De Meern, the Netherlands).

Global DNA methylation - HPLC

DNA was extracted from five livers per dose group except for the 1000 µg/kg bw/d in which only four females were present. Global methylation was analyzed according to Rozhon et al. (2008) and this method has previously been shown suitable to detect small differences in methylation following exposure to different stressors *in vitro* (Bastos Sales et al., 2013). DNA samples (1 µg in 44 µL) were digested to single nucleosides with a mixture of 2.5 mU nuclease P1 and 500 mU DNase I (Sigma, Germany). After addition of 5 µL of 100 mM NaOH, samples were further digested with 1 U alkaline phosphatase (New England Biolabs, Hitchin, UK), in order to obtain the deoxyribonucleosides. Deoxycytidine (dC) and 5-methyl deoxycytidine (5mdC) were quantified with an HPLC-UV system (Shimadzu) equipped with a 125 x 4 mm nucleosil 100–10 SA column (Macherey-Nagel) and a mobile phase consisting of 40 mM acetic acid in 15% acetonitrile (pH 4.8) at a flow rate of 0.6 mL/min. Global methylation status was determined by integrating digested DNA samples on standard curves of both dC and 5mdC (MP Biomedical, France). Percentage methylation was calculated with [5mdC]/([dC] + [5mdC]) x 100.

Full dose range data were analyzed for a statistically significant dose-response using the benchmark dose approach (Slob, 2002) with the PROAST software version 38.1 (www.rivm.nl/proast). Clustered analysis of individual animals from the same litter was applied.

Genome-wide methylation - DREAM assay

Samples of 4 control (from 3 litters) and 4 BPA-exposed females (from 2 litters; 3000 µg/kg bw/d) were analyzed by DREAM (digital restriction enzyme analysis of methylation) at the Temple University School of Medicine (Philadelphia, PA, USA) according to the methods described in Jelinek et al. (2012). In short, this assay was used to identify methylation at specific CpG sites throughout the genome. Genomic DNA was sequentially digested by SmaI and XmaI, which both recognize the sequence CCCGGG. SmaI is methylation sensitive, whereas XmaI is methylation insensitive. Distinct signatures, 5'-GGG at unmethylated sites or 5'-CCGGG at methylated sites were created, and ultimately next generation sequencing was used to map these sites to the genome.

Methylation ratios for each individual CCCGGG site were calculated as a proportion of methylated counts to the sum of unmethylated and methylated counts, and subsequently adjusted for differences in restriction enzyme efficiency using values obtained from spiked in standards. After DREAM analysis, corrected methylation levels were available for 173460 sites mapped to the mouse genome version mm9. Of these sites 17600 had at least 10 counts in every sample and a median sample count of at least 50. Data for these sites were considered as sufficiently informative to be used in subsequent analyses. As an indication, the overall average of counts for all samples and targets was 356 ± 299, so generally well above the informative limit of 10.

All statistical analyses were performed in R statistical software (www.r-project.org/). Methylation levels for control and BPA-exposed mice were compared using a Student's t-test, and statistically significant differences confirmed with a nested ANOVA accounting for intra- and inter-litter variations.

Target methylation - bisulfite pyrosequencing

The EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA) was used for bisulfite treatment of DNA samples according to the manufacturer's instructions. Every DNA sample had a starting quantity of 500 ng and was eluted in 14 µL M-Elution buffer. A premixed methylated calibration standard was used as control (EpigenDx, Worcester, MA, USA).

PCR primers and sequencing primer were selected using PyroMark Assay Design SW 2.0 Software (all PyroMark products are from QIAGEN; sequences in Table 2). Pyrosequence fragments were designed to verify the exact CpGs detected by the

DREAM assay. Oligonucleotides were purchased from Life Technologies (Carlsbad, CA, USA). 2.5 μL of bisulfite-treated DNA was amplified by PCR using the PyroMark PCR Kit in a total volume of 50 μL according to manufacturer's instructions and cycling protocol: 95 °C for 15 min, followed by 50 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s and a final extension at 72 °C for 10 min. Single-stranded template from 20 μL of the biotinylated PCR product was prepared for pyrosequencing analysis using streptavidin-coated Sepharose beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), annealed to 0.375 μM sequencing primer and using a PyroMark Q24 Vacuum Workstation according to instructions supplied by the manufacturer. The primed template was sequenced using PyroMark Gold Q24 Reagents with a PyroMark Q24 System and the assay design generated by PyroMark Assay Design Software was used to create the assay setup with PyroMark Q24 Application Software. Criteria used for the pyrogram were symmetric peaks (no irregularities or side-peaks) and absence of significant signals at positions for control of bisulfite treatment and for control nucleotides dispensed to check for unspecific background. Methylation levels for control and BPA-exposed mice were compared using a nested ANOVA.

Results

Global DNA methylation - HPLC

HPLC was used to assess global DNA methylation levels. The data set, with or without exclusion of the control group, did not show a statistically significant dose-response in female liver (Figure 1). Average group values for methylation ranged from 3.66% to 3.92% and showed a coefficient of variation between 0.013 and 0.071.

New targets - DREAM assay and pyrosequencing

The DREAM assay was used to identify methylation levels at specific CpG sites throughout the genome. An overall distribution analysis showed a large peak at unmethylated targets and a smaller peak at highly methylated targets, without difference between control and BPA-exposed samples (Figure 2A). Overall, there was less variation at strongly methylated or unmethylated sites (Figure 2B), where it should be noted that this difference is not produced by a variation in counts in the assay. The BPA and control groups showed diverging regression curves, suggesting less variation in methylation in BPA-exposed compared to control animals (Figure 2B).

Table 2. PCR and sequence primers used in pyrosequence assay

Target	Primer	Sequence (5' to 3')	PCR product (bp)
Cxcr4	Forward	TGTAGTAATGGGGATGGAATTT	165
	Reverse	TCCATCTTTTTATTACACCCTACAATC*	
	Sequencing	AGGTATGTATTTTATTATTTGAGAT	
Padi4	Forward	GTAGTTTTGGAAATTGGTTGTGTTTA	84
	Reverse	ACTATAACCAATATACACTCCTTAACTC*	
	Sequencing	ATTGGTTGTGTTTAGTTT	
Rbp4	Forward	GAGAAAGTTGGAAGTTAAGGAAAGTTAAAT	156
	Reverse	ACTTCATAACCTACATAATATCCCTATCT*	
	Sequencing	GGGGTTTTTTGGTGA	
Dusp5	Forward	GGTGGGATTTTGGAAGTAATAG	219
	Reverse	ATAAAATACTCCTTCCTACAACATCTA*	
	Sequencing	GGAATTGTTTGAGTTGTATT	
Palm	Forward	AGTTTTTGATAGTGGGGGGTTA	109
	Reverse	AACCAAAAACAACCCTTTCATCT*	
	Sequencing	TAGTGGGGGGTTAGG	
Scube3	Forward	TTGTTAGGGGTGGAAAGATATTAG	170
	Reverse	AAAAATCATAATTACCACCTCCAACAC*	
	Sequencing	ATTTATTTAATATTGGGGTTTTTT	

bp, base-pairs
* biotin labelled primer

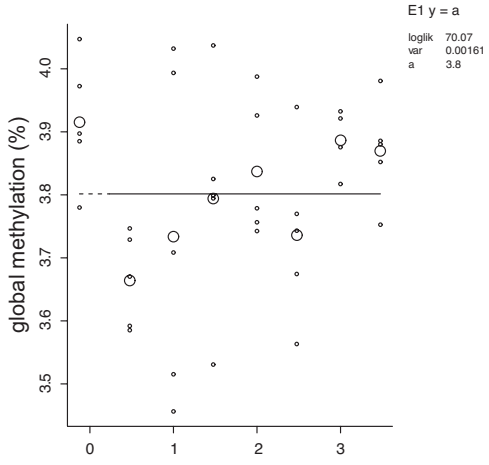

Figure 1. Global DNA methylation in liver of adult female mice perinatally exposed to BPA (3–3000 µg/kg bw/d). Analysis at 23 weeks of age did not result in a significant dose-response for global DNA methylation. Loglikelihood (*loglik*) and variance (*var*) are parameters of significance and data variability. Small symbols: individual mice, large symbols: geometric mean (per dose). The analysis was done with PROAST version 38.1.

Bioinformatic analysis revealed 4 sites with a difference in methylation > 20% and 12 sites with a *p*-value < 10^{-4}. Only one site was found to overlap between these lists, which was located in the vicinity of the *Chordc1* gene. However, this site is located in a Line1 repeat element. Closer inspection of the data revealed that – presumably due to the repeated nature of this site – the majority of the reads for this site were mismapped low quality reads. This site was therefore not taken into account in further analyses and validation steps. By relaxing the stringency criteria to limits of informative value and reliability of 10% difference in methylation and $p < 10^{-2}$, respectively, 19 sites were identified (Table 3). Only three of these 19 sites had relatively low false discovery rates (FDRs) (0.065–0.149), whereas the other 16 had high FDRs (0.243–0.570). Comparison of average methylation per group per locus revealed that a 10% difference is a sufficiently large enough effect size to exceed normal variation (Figure 2C). A heatmap of these 19 selected sites shows the individual methylation levels (Figure 2D), visualizing that highly methylated sites (>50%, top half) in control samples were generally demethylated (except for *Atp2b2*), whereas sites with low methylation in control samples (<50%, bottom half) generally showed higher methylation in exposed samples (except the bottom two). For all 19 CpG sites, the closest neighboring genes were identified (listed as gene A and gene B in Table 3), which resulted in a total of 25 genes, since 6 of the 19 sites were not within a gene but between two genes. Next, we performed a literature search using the keywords BPA, estrog*, and methyl* which produced support for functional involvement of 4 of these genes in BPA phenotypical programming, namely *Cxcr4*, *Padi4*, *Rbp4*, and *Dusp5* (Table 3). The corresponding CpG sites were selected for validation with bisulfite pyrosequencing based on hits for the neighboring gene(s). Assays for these 4 targets and validation showed no differences in DNA methylation at these sites (Figure 3). To further expand our validation, we selected two extra targets based on smallest *p*-value, *Palm* and *Scube3* (Table 3). *Palm* and *Scube3* also did not show any differences (Figure 3). The overall range of DNA methylation was consistent between the DREAM assay and pyrosequencing for the loci tested. When compared to pyrosequence results, differences between control and BPA samples in the DREAM were mostly due to a lower methylation, in either of the two groups.

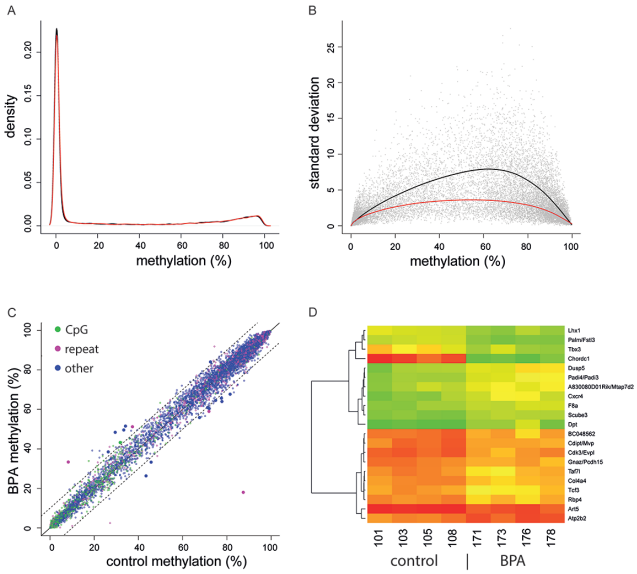

Figure 2. Genome wide restriction sensitive DNA methylation analysis in livers of adult female mice perinatally exposed to 3000 μg/kg bw/d BPA (DREAM assay). (A) Density plot showing data distribution for percentage methylation in control (*black*) and BPA-exposed females (*red*). (B) Comparison of average percentage methylation versus standard deviation per CpG site in BPA-exposed or control females (n = 4, both groups in gray dots). Lines indicate LOESS-smoothed running average for untreated (*black*) and BPA-exposed females (*red*). (C) Scatter plot showing average percentage methylation per group per CpG site in BPA-exposed versus control females. The solid line indicates unchanged levels, dotted lines 10% difference in methylation. Dot colors indicate type of CpG site (green, CpG islands; red, repeats; blue, other [non-island, nonrepeat CpG sites]). Big dots, selected 19 sites with $p < 0.01$ and difference > 10%. (D) Heatmap for 19 sites with $p < 0.01$ and difference > 10%, showing percentage methylation per individual mouse (*left*: 4 control females; *right*: 4 BPA-exposed females (3000 μg/kg bw/d); number indicates animal identifier). The color range indicates percentage methylation along a scale from green (0%) via yellow (50%) to red (100% methylation). The 19 CpG sites in this heatmap are listed and explained in Table 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3. Differentially methylated DREAM targets with more than 10% difference (control-exposed) and $p < 0.01$

Chr	Position	Meth con (%)	Meth BPA (%)	ΔMeth (%)	*p*-value	FDR	Gene A	Gene B	CpGi	Repeat Family	Gene function
9	17813630	87.39	18.04	–69.35	1.48E–05	0.065	*Chordc1*^	*Chordc1*		L1	Stress response
10	79257772	43.4	26.38	–17.03	3.36E–05	0.102	*Palm*#	*Fstl3*			Development (e.g. adrenal gland), transcription
17	28275915	26.33	38.5	12.17	1.86E–04	0.149	*Scube3*#	*Scube3*			Calcium ion binding
4	140327119	33.32	49.61	16.28	5.53E–04	0.243	*Padi4**	*Padi3*			Immune response, transcription, histone citrullination, chromatin modification
11	84474490	45.71	33.31	–12.4	2.25E–03	0.426	*Lhx1*	*Lhx1*			Development, DNA binding
X	70473818	31.71	43.25	11.54	2.32E–03	0.426	*F8a*	*F8a*	yes		Unknown
1	82493890	71.25	60.96	–10.29	2.79E–03	0.435	*Col4a4*	*Col4a4*			Extracellular matrix constituent
11	116083142	81.46	67.72	–13.74	3.02E–03	0.443	*Cdk3*	*Evpl*			Cell cycle, dermal system
1	166659912	8.23	33.38	25.15	3.60E–03	0.46	*Dpt*	*Dpt*		L1	Cell proliferation
10	74010608	74.9	64.56	–10.34	3.77E–03	0.463	*Gnaz*	*Pcdh15*			Intracellular signal transduction, sensory perception
9	108348250	77.73	64.02	–13.72	4.31E–03	0.475	*BC048562*	*BC048562*			Unknown
10	79914949	67.57	55.89	–11.67	4.42E–03	0.475	*Tcf3*	*Tcf3*			Immune response, histone acetylation, transcription, DNA binding
19	38191766	72.01	60.46	–11.55	5.15E–03	0.475	*Rbp4**	*Rbp4*			Gluconeogenesis, glucose homeostasis, development (e.g. eye)
6	113852601	72.57	84.07	11.5	5.77E–03	0.488	*Atp2b2*	*Atp2b2*			Calcium homeostasis, locomotion
1	130492986	29.47	48.41	18.94	6.09E–03	0.503	*Cxcr4**	*Cxcr4*			Immune system, brain development
7	134133041	77.5	65.67	–11.83	6.94E–03	0.533	*Cdipt*	*Mvp*			Lipid metabolism, ErbB signaling
X	155946566	37.27	51.25	13.99	6.96E–03	0.533	*A830080D01Rik*	*Mtap7d2*		ERVK	Unknown
19	53632116	34.13	51.56	17.43	8.21E–03	0.559	*Dusp5**	*Dusp5*			Transcription
X	131009849	71.88	59.02	–12.86	8.88E–03	0.57	*Taf7l*	*Taf7l*	yes	Low_ complexity	Transcription

Table 3. Continued

Table is sorted by FDR (false discovery rate). Chr, Chromosome on which the SmaI site is present; position, position of site on the chromosome; meth con/BPA, average methylation value of control and BPA-exposed females respectively; Δmeth, difference in average methylation between BPA-exposed and control females; gene A/B, the nearest gene (A, up- or downstream) and the second nearest gene (B, generally, but not necessarily, in the opposite direction of gene A) to the SmaI site; CpGi designates whether the SmaI site is found in a CpG island; L1, Line1 element: long interspersed nuclear element; ERVK, mouse endogenous retrovirus K; gene function, most relevant key terms associated with gene A and/or gene B from the National Center for Biotechnology Information (NCBI).

* Genes for follow-up with bisulfite pyrosequencing analysis after hits with literature search using the keywords: BPA, estrog*, methyl*.

Genes for follow-up with bisulfite pyrosequencing analysis based on lowest *p*-value.

^ Majority of the reads for this site were mismapped low quality reads probably due to the Line1 element and this site was therefore excluded for further analysis.

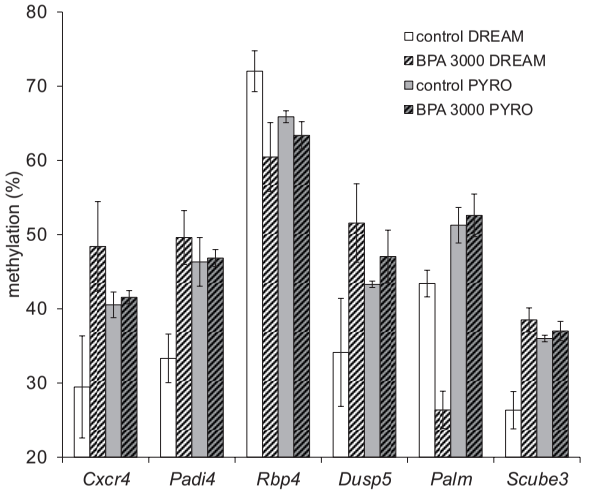

Figure 3. Validation of selected DREAM targets with bisulfite pyrosequencing in livers of adult female mice perinatally exposed to 3000 µg/kg bw/d BPA. Differences in methylation observed in DREAM assay for 6 targets were not seen with bisulfite pyrosequencing. These 6 DREAM targets resulted from (A) cut-offs $p < 0.01$ and difference in methylation > 10%, and (B) an association with literature keywords: BPA, estrog* or methyl* (*Cxcr4, Padi4, Rbp4, Dusp5*) or the smallest *p*-value (*Palm* and *Scube3*; Table 3). DREAM/PYRO, DNA methylation results for CpG site in DREAM assay and with bisulfite pyrosequencing, respectively.

Discussion

In our previous study with BPA exposure during gestation and lactation (van Esterik et al., 2014), we observed a perturbed metabolic phenotype in females. The BPA-induced phenotype occurred during early life, soon after termination of the actual exposure, and persisted thereafter, suggesting that regulation of metabolic functions was disrupted (Vickers, 2014). Permanent epigenetic modifications such as changes in DNA methylation are the probable mechanism of disrupted programming (Jirtle and Skinner, 2007). Therefore, we studied DNA methylation as a potential mark of epigenetic mechanisms related to the BPA-induced metabolic phenotype in female offspring. We analyzed global DNA methylation as a first screen, followed by site-specific analysis of DNA methylation on a global scale with the DREAM assay and verification of the resulting differentially methylated CpG sites by bisulfite pyrosequencing.

No effect of global DNA methylation was detected in the BPA exposure dose range, and there were no apparent factors, such as a deviating control group, to explain the absence of an effect. Therefore, we also employed a different approach to detect effects on DNA methylation. The DREAM assay is a robust, cost-effective alternative for genome-wide DNA methylation screening (Jelinek et al., 2012). Our analysis revealed a number of general characteristics of methylation in the liver of female mice perinatally exposed to 0 or 3000 μg BPA/kg bw/d. Overall, the lower intra-group variation at strongly methylated or unmethylated sites compared to sites in the middle range of methylation (around 50%) may reflect higher dynamics of intermediate methylation compared to the extremes. The observed lower variation in the BPA group means that this exposed group is overall more homogenous. This may suggest that BPA exposure induced a drift of methylation into a specific pattern, although the low number of replicates (n = 4) and the different number of litters in the two groups limit conclusions on the significance or biological relevance of this difference.

The 19 CpG sites identified with the DREAM assay that showed a relevant difference between BPA-exposed and control females either had a low statistical significance, a small size in methylation difference, a high FDR, or any combination thereof. Analysis of functions indicated involvement of these genes in various processes but only two out of 25 targets (*Cdipt* and *Rbp4*) showed a link with metabolism (Table 3), providing no support for a targeted disruption of metabolic functions in the

liver. In addition, none of these 19 CpG sites and associated genes were found in similar studies (Table 1), which does not provide confirmation of our results, anyway concerning liver targeted studies. Support from literature for the relevance of the gene near or containing the observed target CpG site was an additional asset. In this way, *Rbp4*, but not *Cdipt*, was associated with the keywords BPA or estrog* or methyl* (with estrog* included to cover the estrogenic activity of BPA). Still, the sites that performed best according to the above mentioned criteria, including *Rbp4*, were selected for validation, representing a pragmatic compromise.

The methylation differences for the initial set of 4 sites in the DREAM (11–19%) exceeded the technical detection limit of the pyrosequence, and thus were potentially large enough for successful validation. Both the overall extent of DNA methylation and the direction of trend in methylation were consistent between DREAM and pyrosequence assay across the included sites. However, validation of these 4 sites by pyrosequencing did not confirm the methylation differences found by DREAM analysis. The two additional sites, selected for a low *p*-value, were also not validated by pyrosequencing.

The absence of detectable differences in DNA methylation may represent a true absence, analytical shortcomings, or other methodological factors. For instance, although probably representative of events over the whole genome, DREAM targets a selection of CpGs, and effects in non-selected CpGs may thus remain undetected; other high density (sequencing) methods may reveal other targets. The choice of the target organ is another factor. The liver was selected for analysis because of its role in energy and xenobiotic metabolism (Sevior et al., 2012) and because of its relative homogeneity. Although hepatic parenchymal cells are the major cell population in the liver, many other cell types are present that may mask effects in the parenchyme, and even hepatocytes in various lobule zones exhibit different functions. The liver may thus not be that homogenous after all, and detection of small effects may need a more focused approach. Furthermore, liver may not be a primary target organ of disrupted programming by BPA. Other organs like the hypothalamus and pancreas islets may be more informative than the liver in this respect, because these are central players in the control of energy homeostasis. However, the explorative design of this study did not allow for screening of such small and inaccessible tissues. Also, our assumption that liver tissue could be a good proxy for methylation changes programmed early in life, because such changes should be distributed in any tissue through clonal inheritance,

may not hold true if specific BPAinduced methylation changes in developing cells were obscured by further differentiation events.

The absence of effects on DNA methylation in our study deviates from observations by others, as summarized in Table 1. Differences may be explained by experimental conditions including BPA dose, administration route, species and strain, sex and tissue selected for analysis and other experimental factors such as background diet (methyl donors) and microbiome. These experimental conditions and factors are all potential determinants for the observed effects of the toxicant on DNA methylation. In that perspective, most studies in Table 1 either measured at relatively early ages, or applied continuous exposure. Only the studies of Ma et al. (2013), Li et al. (2014) and O'Brien et al. (2014) are comparable to our study with measurement of DNA methylation long after termination of actual exposure in order to reveal permanent programming effects, where the obvious differences with our study are in species (rats) and sex (males) or in tissue (bone-marrow derived mast cells) and method of analysis. A recent study demonstrated non-monotonic effects of perinatal BPA exposure on liver DNA methylation (Kim et al., 2014), that cannot be accounted for in our study in which only one relatively high dose was analyzed in the DREAM assay. Interestingly, in this study epigenetically dysregulated pathways by perinatal BPA in liver included metabolism (Kim et al., 2014).

While DNA methylation changes after early BPA exposure is the most well studied epigenetic mechanism (Stein, 2012), other *in vitro* and *in vivo* studies have also found BPA to affect epigenetics by inducing histone modifications (Bhan et al., 2014; Dhimolea et al., 2014). In addition, BPA could also disrupt noncoding RNA-mediated signaling pathways (e.g., microRNAs, small interfering RNAs) which regulate both DNA methylation and histone modifications (Singh and Li, 2012). Two *in vitro* studies with mouse (Cho et al., 2010) and human (Avissar-Whiting et al., 2010) cell lines show alterations in microRNAs after BPA exposure. Changes in epigenetic mechanisms other than DNA methylation may explain the altered metabolic phenotype in our adult female mice. Taken together, a significant number of studies show that perinatal BPA exposure can affect the epigenome of rodents regardless of experimental conditions. Especially studies that indicate that BPA-induced epigenetic changes can be transgenerationally inherited (Li et al., 2014; Yan, 2014) warrant further research.

In conclusion, literature provides ample indications that exposure to BPA early in life can modify the epigenome. We could not confirm this in liver in our BPA-exposed female F1 mice using a number of methods to analyze DNA methylation on various levels, possibly due to different experimental conditions or use of non-target tissue. Overall, analysis of DNA methylation in the liver in adult female offspring did not provide an explanation for the observed altered metabolic phenotype resulting from exposure to BPA early in life.

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